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Production of an Oligosaccharide-specific Cellobiohydrolase from the Thermophilic Fungus *Thielavia terrestris*

James Sy-Keen Woon (astrowoon@yahoo.com) [1], Mukram Mohamed Mackeen (mukram@ukm.edu.my) [2, 3], Amirul Husna bin Sudin (amirulhusna55@gmail.com) [2], Nor Muhammad Mahadi (nor.mahadi@gmail.com) [3,4], Rosli Md Illias (r-rosli@utm.my) [5], Abdul Munir Abdul Murad (munir@ukm.edu.my) [1], Farah Diba Abu Bakar [1*]

[1] School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

[2] School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

[3] Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.

[4] Malaysia Genome Institute, Jalan Bangi Lama, 43000 Kajang, Selangor Malaysia.

[5] Department of Bioprocess Engineering, Faculty of Chemical Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia.

* Corresponding author: Email: fabyff@ukm.edu.my

Abstract

Objectives To express and determine the hydrolytic activity of a cellobiohydrolase (TTCBH6B) from the thermophilic fungus *Thielavia terrestris* in *Pichia pastoris*. *Results Ttcbh6B* encodes 507 amino acid residues and has a predicted molecular mass of 54 kDa. TTCBH6B contains a familial 6-glycosyl hydrolase catalytic domain and a type I carbohydrate-binding module (CBM). TTCBH6B was expressed and purified to homogeneity but the purified enzyme was inactive against Avicel, a common CBH substrate. Nonetheless, TTCBH6B was able to digest Celluclast-treated Avicel producing cellobiose (0.27 μmol min^{-1} mg^{-1}) that was detected by thin-layer chromatography and quantified via DNS reagent method. To determine the substrate preferences of TTCBH6B, oligosaccharides of varying numbers of subunits were generated by acid hydrolysis of Avicel and fluorescently tagged. Peaks corresponding to oligosaccharides containing three to six glucose units were reduced to cellobiose after addition of TTCBH6B. *Conclusion* TTCBH6B is active against shorter oligosaccharides rather than polysaccharides.

Keywords: Avicel, carbohydrate-binding module, cellobiose, Celluclast, endoglucanases, glycosyl hydrolases, 2-aminobenzamide

Introduction

Cellobiohydrolases (EC 3.2.1.91) are major components of the fungal cellulase system which includes cellobiohydrolases, endoglucanases and β-glucosidases. Together, these enzymes synergistically cleave celluloses into glucose subunits. Cellobiohydrolases (CBHs) act from the opposite ends of cellulose chains releasing cellobioses (Teeri 1997), a capacity that has made them the instrument of choice in industrial hydrolysis of cellulosic biomass containing highly crystalline regions (Abdeljabbar et al. 2012). Unfortunately however, their low specific activities at higher temperatures (Voutilainen et al. 2009) restrict their use driving the search for thermostable enzymes (Yeoman et al. 2010).

Based on the sequence similarities documented in the CAZy database (http://www.cazy.org/) (Henrissat and Bairoch 1996), fungi generally produce two families of CBHs - the glycosyl hydrolase family 7 and the glycosyl hydrolase family 6. As cellulases and hemicellulases of the thermophilic fungus *Thielavia terrestris* have outstanding catalytic and stability characteristics (Berka et al. 2011), we sub-cloned a member of the glycosyl hydrolase family 6 and expressed the construct (*ttcbh6b*) in the methylotrophic yeast *Pichia pastoris* which secretes large amounts of proteins under control of the alcohol oxidase (*AOX*1) promoter (Kamaruddin et al. 2015).

Materials and methods

Microbial strains

Escherichia coli DH5α (Promega, WI, USA) was used as the bacterial host for all cloning procedures. *Pichia pastoris* strain X-33 (Invitrogen/Life Technologies, NY, USA) was used for the expression of *T. terrestris cbh*6B. Preparation of media and growth of the yeast were performed following the instruction manual of Invitrogen/Life Technologies *Pichia* expression system.

Gene synthesis and sub-cloning of *ttcbh*6B

Codons in the ORF of *ttcbh*6B (NCBI gene ID: 11521362) were optimized for expression in *P. pastoris* and synthesized by Invitrogen/Life Technologies. *Cla*I and *Xba*I restriction adaptors were added to the 5'- and 3'-ends respectively and used to insert a sub-clone of *ttcbh*6B into the expression vector pPICZαC (Invitrogen/Life Technologies). Manipulation of DNA was carried out using standard procedures according to Sambrook et al. (2001).

Transformation of *P. pastoris* X-33

Transformation of *P. pastoris* X-33 was performed as according to the instruction manual of Invitrogen/Life Technologies *Pichia* expression system using an Eppendorf Electroporator Model 2510 (Eppendorf, Hamburg, Germany) at 1,500 Volts. Positive transformants were confirmed by PCR (Lõoke et al. 2011) using a pair of *AOX*1 primers targeting the insert flanked by the *AOX*1 gene. Transformants containing multiple insertions were selected on YPDS (Yeast Extract Peptone Dextrose medium plus Sorbitol) plates containing various concentrations of Zeocin (500 μg ml^{-1}, 1,000 μg ml^{-1} and 2,000 μg ml^{-1}).

Expression of *ttcbh*6B

Transformants carrying the expression construct pPICZαC_*ttcbh*6B were grown in 50 ml of buffered glycerol complex medium at 30 °C to an OD_{600} of 2 to 3. Then the cells were centrifuged for 5 min at 1,500×*g* and re-suspended in 25 ml of buffered methanol complex medium. Methanol was added every 24 h to a final concentration of 1.0% (v/v) during 72 h incubation (at 30 °C with shaking at 240 rpm). Culture supernatants were clarified by centrifugation (5 min at 3,000×*g*) and stored at −20 °C. TTCBH6B production was verified by SDS-PAGE (12% polyacrylamide) followed by western-blot analyses using mouse anti His-tag monoclonal antibodies (Novagen, WI, USA) and a HRP-conjugated anti-mouse antibodies (Promega) for chemi-luminescent detection on X-ray films. Protein concentrations were determined using the Bradford method (Bradford 1976).

Purification of TTCBH6B

Culture supernatants containing His-tagged TTCBH6B recombinant enzymes were purified by immobilized metal-ion affinity chromatography (IMAC) using a 1 ml HiTrap chelating column charged with Ni^{2+} (AKTA prime system from GE Healthcare Bio-Sciences Corp., NJ, USA). The column was equilibrated with 10 ml binding buffer containing 20 mM of imidazole. This was followed by loading 1 ml of crude protein and eluting the proteins with a gradient of imidazole ranging from 100-300 mM. Eluted fractions that contained high concentrations of proteins were pooled, diluted two-fold with 50 mM sodium acetate buffer (pH5) and subsequently concentrated using Vivaspin centrifugal concentrators (cut-off of 10 kDa - GE Healthcare Bio-Sciences Corp.).

Enzyme assays and thin-layer chromatographic (TLC) analyses

Enzyme assays were performed in 400 µl reaction mixtures in 30 mM sodium acetate buffer (pH 5) and 1% (w/v) Avicel PH-101 (Sigma-Aldrich Corp, MO, USA) at 50 ºC containing 5 µg purified TTCBH6B. Mixtures were incubated for 1 h with agitation at 1,000 rpm. Assays were also performed using the soluble synthetic substrate 4-methylumbelliferyl β-D-cellobioside (MUC), 4-nitrophenyl β-D-cellobioside (pNPC) and 4-nitrophenyl β-D-lactopyranoside (pNPL) (Sigma-Aldrich Corp) as described in Woon et al. (2015). To screen for optimum hydrolytic conditions, assays were performed at different pHs (pH 3-10) and temperatures (30 ºC, 40 ºC, 50 ºC, 60 ºC and 70 ºC).

To produce shorter oligosaccharides as possible substrates for TTCBH6B, Avicel was pre-treated with 20 µg of the commercial cellulase complex Celluclast 1.5 L (Novozymes, Bagsvaerd, Denmark) at 50 ºC for 2 h with agitation at 1,000 rpm. The hydrolysed product was analysed on thin-layer chromatography plates (Silica Gel 60 F_{254} from Merck Millipore, Darmstadt, Germany) using a 10 ml mobile phase containing butanol: ethanol: water (7:2:1 v/v/v). The separated compounds were visualized by spraying with Orcinol [1 g l^{-1} Orcinol dissolved in 5 % (v/v) sulfuric acid]. To be an effective substrate for TTCBH6B, the cellobiose produced by Celluclast first needs to be removed by digesting cellobiose into glucose with 20 µg of cellobiase (Novozyme 188, Novozymes)(incubation at 50 ºC, 2 h, 1,000 rpm agitation). Afterwards, the Celluclast and Novozyme 188 were denatured by boiling for 15 min. When cooled, 10 µg of TTCBH6B was added to the reaction mixture and incubated for 30 min (at 50 ºC, 1,000

rpm agitation). Amounts of cellobiose were estimated using the DNS reagent method (Miller 1959) with three replications.

Generation of fluorescent-tagged oligosaccharides as potential substrates

Oligosaccharide (glycan) analysis was carried out using standard methods in glycomics based on high performance liquid chromatography (HPLC)-fluorescence detection and glucose unit (GU) analysis. Oligosaccharides of various degrees of polymerization were generated by hydrolyzing Avicel (30 mg) in 150 µl of 0.1 M HCl for 1 h at 100 ^{o}C. To remove the acid afterwards, the reaction mix was passed through a column containing 75 µl Bio-Rad AG2-X8 resin (Bio-Rad, CA, USA) and washed through with 450 µl distilled water. The hydrolyzed product was dried using a Büchi rotary evaporator Model R200 (Büchi, Essen, Germany) and re-suspended in 50 mM pH 5 acetate buffer. TTCBH6B (10 µg) was then added to the oligosaccharides at 50 ^{o}C for 30 min. Afterwards, TTCBH6B was denatured by boiling for 15 min and the enzyme was removed by passing the reaction mix through the Spe-ed2 Amide-2 column (Applied Separations, PA, USA) and eluted with 300 µl of distilled water. Then, the products were freeze-dried and labelled with 2-aminobenzamide (2-AB) by adding 20 µl of the 2-AB reagent (10 mg 2-AB + 50.27 mg sodium cyanoborohydride in 800 µl of DMSO/Acetic acid, 7:3 v/v) to the dried samples and incubated for 2 h at 70 ^{o}C. The extracted samples were dried using a SpeedVac (Thermo Fisher Scientific, MA, USA) and re-dissolved in 100 µl of 50% acetonitrile (v/v). The samples were then injected into a HPLC machine equipped with a Waters 2475 fluorescence detector set at Exλ 360 nm and Emλ 426 nm. Samples were separated on a XBridge Amide column (4.6 x 250 mm, 3.5 µm particle size) (Waters Corporation, MA, USA) with the following gradient conditions: time – 0 min (t - 0), 86% A, 14% B (0.8 ml min^{-1}); t- 6, 86% A, 14% B (0.8 ml min^{-1}); t- 45, 46.6% A, 53.4% B (0.8 ml min^{-1}); t- 47, 5% A, 95% B (1.0 ml min^{-1}); t- 49, 5% A, 95% B (1.0 ml min^{-1}); t- 51, 86% A, 14% B (1.0 ml min^{-1}); t- 52, 86% A, 14% B (1.0 ml min^{-1}); t- 53, 86% A, 14% B (1.0 ml min^{-1}); t- 60, 86% A, 14% B (0.8 ml min^{-1}), in which solution A contained 80% acetonitrile (v/v) and 20% 100 mM ammonium acetate (w/v) while solution B contained 20% acetonitrile (v/v) and 20% 100 mM ammonium acetate (w/v).

In silico analyses of TTCBH6B

Domains in the amino acid sequence of TTCBH6B were sought using Prosite (http://prosite.expasy.org/). To find the active site, Multiple Sequence Alignments (MSA) were generated using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2) on TTCBH6B and comparing them to known active sites of other CBHs (www.uniprot.org). Modelling of protein homology was performed using the SWISS-MODEL software on Automated Mode (http://swissmodel.expasy.org/) and the coordinates of the *Chaetomium thermophilum* (PDB: 4A05) cellobiohydrolase as template. The stereo-chemical properties of the protein model were validated using the RAMPAGE Server (http://mordred.bioc.cam.ac.uk/~rapper/rampage2.php). Putative *N*-glycosylation sites in TTCBH6B were determined using NetNGlyC 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/).

Results

Transformation of *P. pastoris* X-33

Transformation of *P. pastoris* with the expression cassette yielded > 50 transformants, sixteen of which were randomly picked and re-streaked on YPDS plates containing different levels of Zeocin (500, 1,000 and 2,000 μg ml^{-1}). PCR on colonies of eight randomly selected transformants showed that seven harboured the target gene and were used for enzyme production.

Expression and purification of TTCBH6B

The expression cassette pPICZαC_TTCBH6B encoded 507 amino acids with a calculated molecular mass of 54 kDa and an isoelectric point of 5.09 (http://www.expasy.ch). A transformant that was resistant to 1,000 μg ml^{-1} Zeocin yielded 5.20 g l^{-1} of crude protein and was selected for enzyme production. Western-blot detection methods confirmed the presence of the target protein as an intact protein band (Fig. 1a). The single band of purified TTCBH6B seen in SDS-PAGE (Fig. 1b) indicated an absence of proteolysis.
(Fig.1)

Enzymatic activity of TTCBH6B

Regardless of pH, temperature, assay period and the amount of enzyme, TTCBH6B was inactive toward Avicel and the soluble synthetic substrates (MUC, pNPC, pNPL). In contrast, Avicel incubated with Celluclast yielded two distinct bands on TLC plates with R_f values similar to glucose and cellobiose (Fig. 2). To confirm the identities of the products from the Celluclast incubation, a cellobiase (Novozyme 188) was added to the products of Celluclast-treated Avicel. As a consequence, the band corresponding to cellobiose disappeared while the band corresponding to glucose increased in intensity confirming that the primary products of Celluclast-treated Avicel were glucose and cellobiose. Addition of this substrate to reaction mixtures containing TTCBH6B produced 0.08 µmol cellobiose as detected by TLC with a specific substrate activity of 0.27 ± 0.02 µmol min^{-1} mg^{-1}.

(Fig. 2)

To study the substrate preference of TTCBH6B, we produced a range of oligosaccharides via partial acid hydrolysis of Avicel. The HPLC results showed that oligosaccharides ranging from one to six glucose units (GU) were generated in decreasing amounts after partial hydrolysis (Fig. 3). Addition of TTCBH6B reduced the intensities of peaks corresponding to cellotriose (G3), cellotetraose (G4), cellopentose (G5) and cellohexose (G6) but increased the intensity of the cellobiose peak (G2) by 51% (Table 1). The peaks for G4, G5 and G6 were reduced to baseline levels, suggesting a complete utilization of these substrates within the 30 min incubation period (at 50 ^{o}C)(Fig. 4). The peak corresponding to glucose was however decreased slightly (by 9%)(Table 1).

(Table 1).

(Fig. 3 and Fig. 4)

In silico analyses of TTCBH6B

Domain analyses showed that TTCBH6B contains a glycosyl hydrolase family 6 catalytic domain (CD) and a type-I carbohydrate binding module (CBM). Multiple Sequence Alignments (MSA) with other CBHs of the same family identified three aspartic acids (D211, D257, D436) as the active sites of TTCBH6B.

A homology model (Fig. 5a) of TTCBH6B was constructed using *C. thermophilum* Cel6A (which shares 80 % amino acid sequence similarity) as the template (PDB: 4A05) and the modelled structure evaluated using a Ramachandran plot which fulfilled the criterion of proper distribution of residues in different regions (Ramachandran et al. 1963). About 95 % and 5 % of the residues were present in

favoured and allowed regions respectively. The homology model of TTCBH6B proposed a distorted *α/β*-barrel topology (Thompson et al. 2012) composed of seven *β*-strands that form an incomplete circular structure. Two *N*-glycosylation sites on the catalytic domain were predicted at residues N172 and N440 (represented by green spheres in Fig. 5b).

(Fig. 5)

Discussion

The database of the Joint Genome Institute (JGI) contains four distinct, annotated CBHs of *T. terrestris* - CBH6A, CBH6B, CBH7A and CBH7B (Woon et al. 2015). Domain analyses indicated that TTCBH6B belongs to glycosyl hydrolase family 6 (GH6) that cleaves a disaccharide from the reducing-end of the cellulose chain (Teeri 1997). This might explain the lack of activity toward all soluble substrates tested (MUC, pNPC, pNPL) as they are labelled with either methylumbelliferone or nitrophenol at their reducing ends.

The degrees of polymerization of the commercial microcrystalline cellulose called Avicel range from 130 to 225 glucose units (Terinte et al. 2011). Although TTCBH6B was unable to digest the native Avicel, it was capable of hydrolyzing the residual products of Celluclast-treated Avicel, suggesting that TTCBH6B substrate preference lies towards smaller oligosaccharides. Subsequent analyses using 2-AB-labelled substrates identified cellotriose (G3), cellotetraose (G4), cellopentose (G5) and cellohexose (G6) as potential substrates for TTCBH6B (Fig. 4). The production of cellobiose indicated the cellobiohydrolase-like activity of TTCBH6B. Interestingly, the amount of glucose did not increase over the 30 min incubation period but was elevated by ~50% (Supplementary Figure 2) when incubated for 2 h at 50 ^{o}C. This reflects the dynamics of the hydrolysis process where large oligosaccharides were first cleaved into smaller ones. At the end-point of analysis, all digested products will end up as part of the glucose or cellobiose pool depending on the initial oligosaccharide substrate length whereby a single unit of cellotriose will produce one unit of cellobiose and one unit of glucose, a single unit of cellotetraose will only produce two units of cellobiose, a single unit of cellopentose will produce two units cellobiose and a single unit of glucose; and the cellohexose will produce three units of cellobiose.

Changes to the *N*-glycosylation patterns of cellulase catalytic domains (CDs) can unpredictably affect enzyme activity, binding and stability (Beckham et al. 2012). As TTCBH6B was expressed in a

foreign host, the glycosylation pattern in *P. pastoris* might have changed. Interestingly, one of the predicted *N*-glycosylation sites (N440) resides on the flexible loop that forms the active-site tunnel of TTCBH6B (Fig. 5b) possibly hindering or interfering with substrate binding. Indeed, there are numerous cases of recombinant fungal CBH expression in *P. pastoris* showing decreased activities on insoluble crystalline substrates such as Avicel or bacterial microcrystalline cellulose (Boer et al. 2000; Godbole et al. 1999; Kanokratana et al. 2008).

Interestingly too, another member of CBH from *T. terrestris* (CBH7B) was inactive against Avicel but worked synergistically with the commercial enzyme Cellic CTec2 to degrade oil palm empty fruit bunches thus enhancing saccharification (Woon et al. 2015). Gao et al. (2012) discovered a non-hydrolytic CBH glycoform produced by *Penicillium decumbens* that was inactive against all substrates but worked synergistically with commercial cellulases to hydrolyze cotton fibres. Our approach has been proven useful in profiling the substrate preference of the TTCBH6B which has a pronounced specificity towards shorter oligosaccharides rather than polysaccharides.

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Figure legends

Fig. 1 (a) Western blot detection of TTCBH6B from *P. pastoris* transformants tolerant to different Zeocin levels. Lane 1: T1 (500 μg ml^{-1}), Lane 2: T2 (1,000 μg ml^{-1}), Lane 3: T3 (2,000 μg ml^{-1}). (b) SDS-PAGE of purified TTCBH6B. Lane F41-F47: Fractions of purified TTCBH6B eluted with a gradient of imidazole ranging from 100-300 mM, lane M: molecular weight marker.

Fig. 2 Thin-layer chromatographic (TLC) analyses of the products of TTCBH6B digests of various substrates. Lane 1: glucose and cellobiose controls (1 mg ml^{-1} each), Lane 2: products of Celluclast-hydrolyzed Avicel, Lane 3: products of Celluclast-hydrolyzed Avicel treated with Novozyme 188, Lane 4: products of TTCBH6B hydrolysis of pre-treated Avicel (treated with Celluclast followed by the cellobiase Novozyme 188 to remove cellobiose).

Fig. 3 HPLC chromatograms of acid-hydrolyzed Avicel containing oligosaccharides of various degrees of polymerization. (a) A cropped view featuring all six cello-oligosaccharides (G1-G6) and (b) an enlarged view on the selected region. G1: glucose, G2: cellobiose, G3: cellotriose, G4: cellotetraose, G5: cellopentose, G6: cellohexose.

Fig. 4 Overlain HPLC chromatograms of acid-hydrolyzed Avicel (red), and acid-hydrolyzed Avicel treated with TTCBH6B (black). G1: glucose, G2: cellobiose, G3: cellotriose, G4: cellotetraose, G5: cellopentose, G6: cellohexose.

Fig. 5 Homology models of the TTCBH6B catalytic domain (CD). (a) The distorted α/β barrel topology of TTCBH6B. (b) Van der Waals surface representation of TTCBH6B featuring the putative active sites (red spheres) and two predicted *N*-glycosylation sites (green spheres). The hollow, active-site tunnel is surrounded by the active sites.

Supplementary Figure 1 Overlain HPLC chromatograms of the acid-hydrolyzed Avicel (black), and the acid-hydrolyzed Avicel treated with TTCBH6B (blue), along with glucose (pink) and cellobiose (light blue).

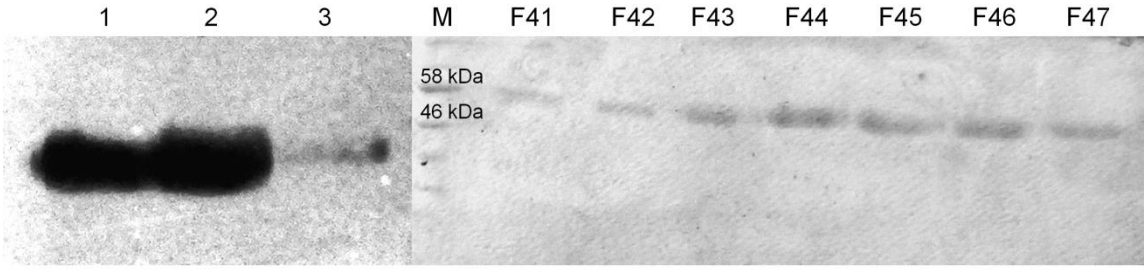
Supplementary Figure 2 Overlain HPLC chromatograms of the acid-hydrolyzed Avicel (red), acid-hydrolyzed Avicel treated with TTCBH6B for 30 min (blue), and acid-hydrolyzed Avicel treated with TTCBH6B for 2 h (black).

Table 1

Relative amounts [in fluorescence emission units - (EU)] of cello-oligosaccharides liberated from the acid-hydrolyzed Avicel (control) and treated samples (acid-hydrolyzed Avicel + TTCBH6B).

Retention time (min)	5.4	7.5	10.3	15.4	21.4	26.7
Oligosaccharide [a]	GU=1	GU=2	GU=3	GU=4	GU=5	GU=6
Peak intensities of hydrolyzed Avicel (EU)	1243 ± 1.1	229 ± 3.8	88 ± 0.4	33 ± 0.1	17 ± 0.6	9 ± 0.1
Peak intensities of hydrolyzed Avicel+TTCBH6B (EU)	1127 ± 26.2	345 ± 7.2	57 ± 0.1	15 ± 0.1	6.3 ± 0.1	3.3 ± 0.1
Change (%)	-9	51	-35	-53	-63	-63

[a] GU= glucose unit. Peaks corresponding to GU= 1 and GU=2 were identified based on overlaying chromatograms containing cellobiose and glucose (Supplementary Figure 1). Subsequent oligosaccharides (GU = 3 to 6) were assigned based on molecular weights and retention times.



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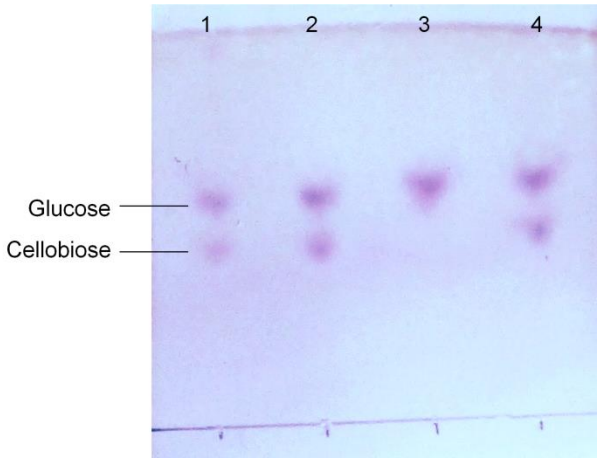

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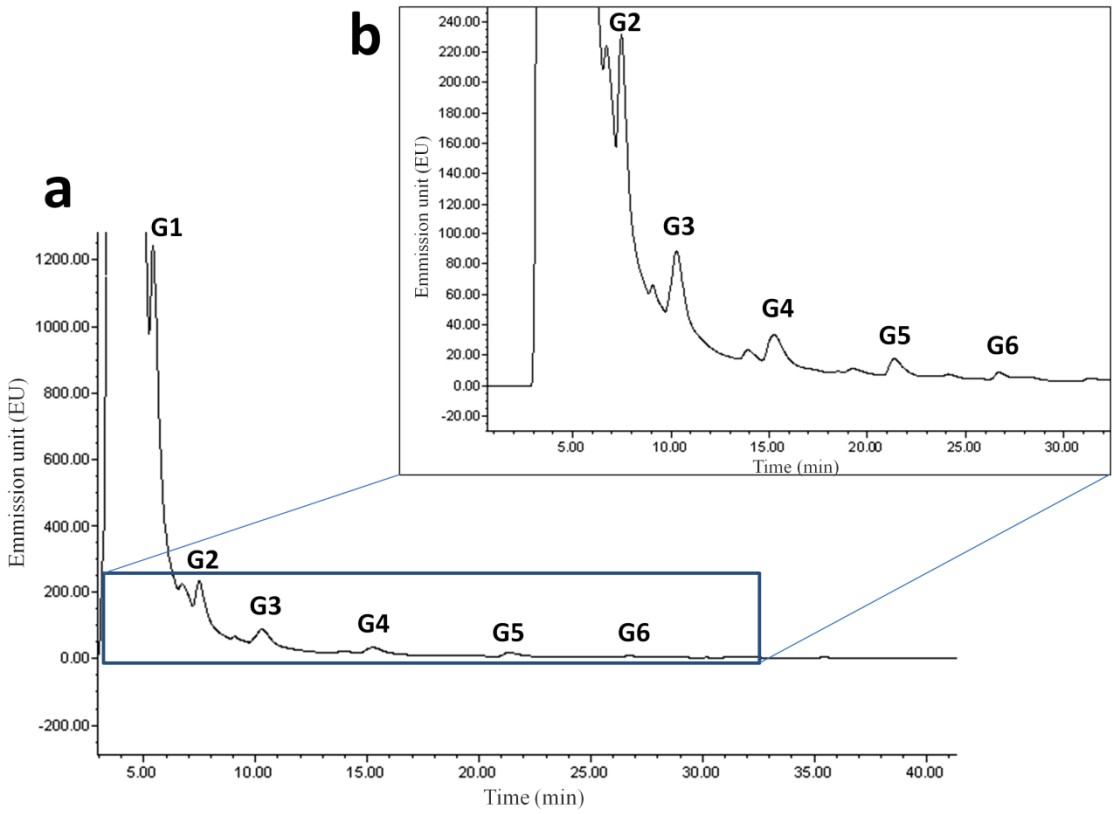

Fig. 3 HPLC chromatograms of the acid-hydrolyzed Avicel containing oligosaccharides of various degrees of polymerization. (a) A cropped view featuring all six cello-oligosaccharides (G1-G6) and (b) an enlarged view on the selected region. G1: glucose, G2: cellobiose, G3: cellotriose, G4: cellotetraose, G5: cellopentose, G6: cellohexose.

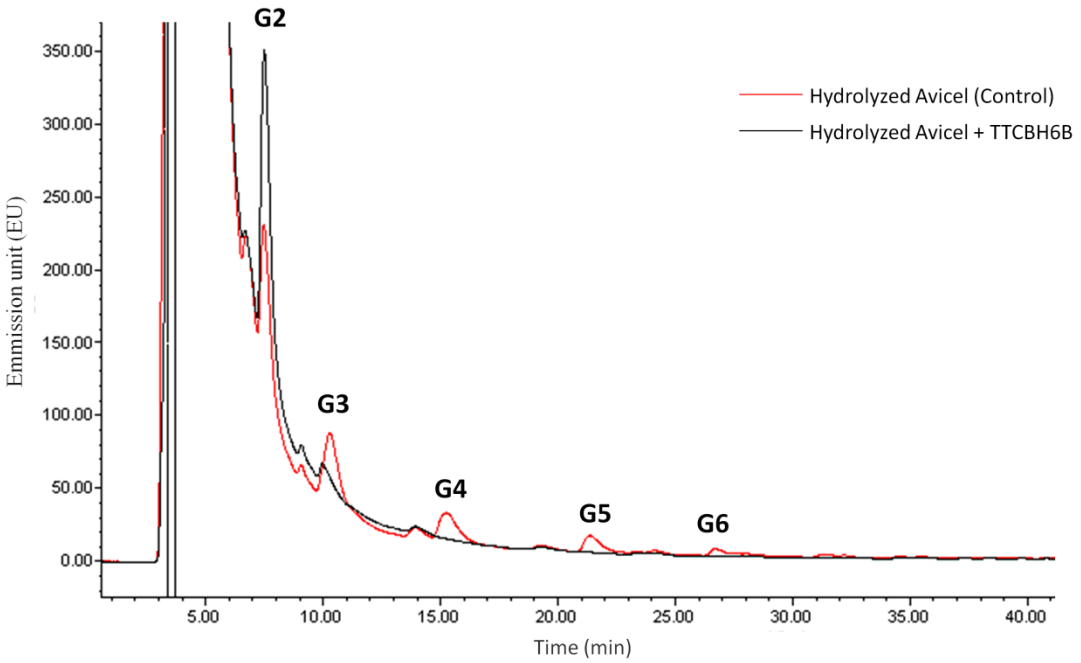

Fig. 4 Overlain HPLC chromatograms of the acid-hydrolyzed Avicel (red), and the acid-hydrolyzed Avicel treated with TTCBH6B (black). G1: glucose, G2: cellobiose, G3: cellotriose, G4: cellotetraose, G5: cellopentose, G6: cellohexose.

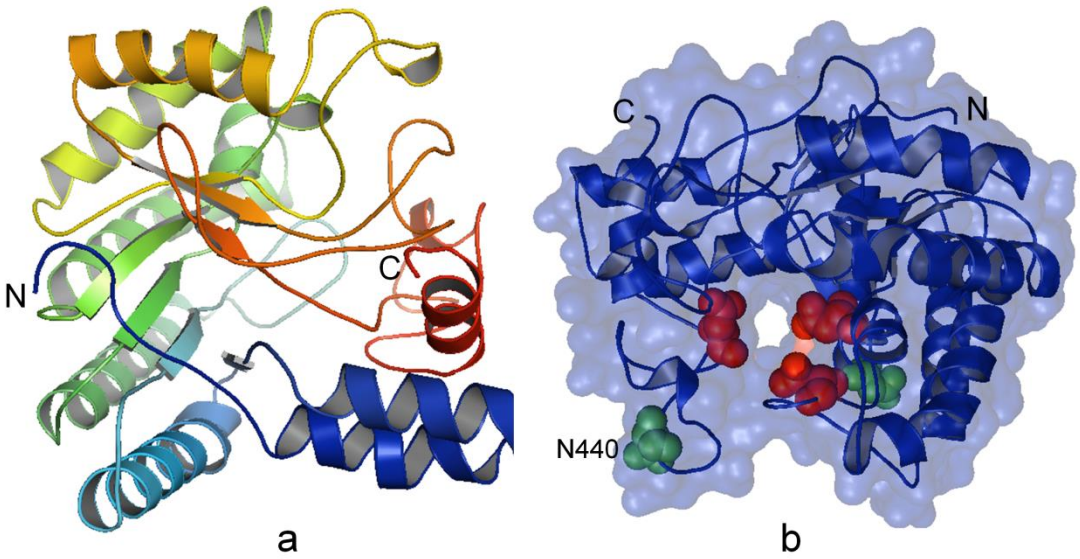

Fig. 5 Homology models of the TTCBH6B catalytic domain (CD). (a) The distorted *α/β* barrel topology of TTCBH6B. (b) Van der Waals surface representation of TTCBH6B featuring the putative active sites (red spheres) and two predicted *N*-glycosylation sites (green spheres). The hollow, active-site tunnel is surrounded by the active sites.